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Purification and functional characterization of recombinant Balsamin, a ribosome-inactivating protein from *Momordica balsamina*

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Abstract: Balsamin, a type I ribosome-inactivating protein (RIP), has been shown to inhibit HIV-1 replication at the translation step. Our recent studies have shown that balsamin also possess anti-tumor, antibacterial and DNase-like activity, however, the amount of natural balsamin in *Momordica balsamina* seeds is limited and preclinical studies require large quantities of pure, bioactive balsamin. Therefore, in this study, we cloned the balsamin gene, expressed it in *E.coli* BL21 (DE3) strain and purified it by nickel affinity chromatography. Functional analysis indicated that balsamin exhibits both RNA *N*-glycosidase activity, releasing the Endo-fragment from rabbit reticulocyte rRNA, and DNase-like activity, converting the supercoiled form of a plasmid into the linear form in a concentration-dependent manner. Analysis of secondary structure revealed that recombinant balsamin mainly consisted of α-helical and random coiled with minimal turns and β-sheets. Recombinant balsamin was found to be stable in the temperature range of 20-60 °C and pH range of 6-9. Antimicrobial assays showed that the minimum inhibitory concentrations of recombinant balsamin for various pathogens ranged between 1.56-12.5 µg/ml. Heterologous expression and purification of balsamin carries great importance as it provides an alternative approach for large-scale preparation of biologically active recombinant balsamin, which is difficult from its natural source.

Keywords: bioactive, nutraceutical, RNA *N*-glycosidase, DNase, antimicrobial and heterologous expression

1. Introduction

Ribosome inactivating proteins (RIPs) are protein synthesis inhibitors that cleave the glycosidic linkage between adenine and ribose in a highly conserved SR (α-sarcin/ricin) loop in the 28S rRNA. This cleavage prevents binding of elongation factors during the translation process, thus inhibiting protein synthesis [1]. RIPs are widely distributed in plants including *Momordica* species. RIPs isolated from *Momordica* species have attracted growing interest for their role in viral and tumor growth inhibition. Moreover, their unique natural role of targeting the conserved host protein synthesis machinery makes them a potential tool in chemotherapy for cells that develop drug resistance [2-4]. Since RIPs bind to specific receptors on the surface of cancer cells, they pose minimal or no detectable adverse effects on normal cells, thus, increasing their potential as anticancer therapeutic agents [3,4,5]. Besides RNA *N*-glycosidase activity, RIPs exhibited polynucleotide adenosine glycosidase, antimicrobial, DNase, and broad-spectrum antiviral activities including against human immunodeficiency virus [6]. RIPs have been exploited in plant protection against various fungal, viral and bacterial pathogens and are therefore also known as antimicrobial proteins [7,8].

Balsamin isolated from *Momordica balsamina* is a type-I RIP that can inhibit HIV-1 replication by targeting the translation step that occurs prior to HIV-1 budding and release [9]. Our recent studies have shown that balsamin also possesses DNase-like activity, inhibits the growth of bacterial pathogens and induces apoptosis in breast cancer cells [10, 11]; however, the amount of balsamin in *M. balsamina* seeds is limited. Moreover, recovery and purification of balsamin from natural sources involves multiple purification steps that compromises protein yield. Heterologous protein expression in a suitable recombinant strain offers an attractive alternative with higher yields, shorter fermentation time and cheaper production costs. Therefore, in this study the balsamin gene was cloned and expressed, and the biological functions of recombinant balsamin (rBalsamin) were investigated.

Several RIP genes from *Momordica* species have been heterogeneously expressed. α-momorcharin (α-MC) was the first gene to be cloned using a λgt11 cDNA library constructed from *M. charantia* seeds, followed by its mass production in a heterologous system, the *E.coli* Rosetta (DE3) pLysS strain [12,13]. The MAP30 gene was cloned in a pET-28 vector and expressed in *E.coli* BL21(DE3) cells [14]. In other studies, Momordica antiviral protein (MAP30) was fused with the human-derived cell penetrating peptide HBD to improve the uptake efficiency by tumor cells and cytotoxicity of MAP30 [15]. In our previous studies, we

investigated various functional properties of balsamin [9-11]. However, to explore animal studies and clinical trials a large quantity of pure protein is required. Thus, in this study we attempted the heterologous expression of balsamin.

Immobilized metal affinity chromatography (IMAC) has been widely used for large-scale purification of proteins linked to polyhistidine residues (His-tag) at the N- or C- terminus. IMAC provides high specificity and ease of purification [16]. The ability of histidine residues to bind to an immobilized metal ion matrix with high affinity under denaturing and native conditions has made the polyhistidine tag a powerful tool for protein purification [17]. The immobilization of a His-tag on an IMAC matrix is a rapid and inexpensive method used to study protein-protein interactions and to purify recombinant proteins with up to 95% purity [18].

Herein we report for the first time, heterologous expression of His-tagged rBalsamin in an *E.coli* expression system. Of all the expression systems, *E.coli* was used for this study because of its advantage over other host cells, namely fast growth kinetics, ease of transformation with exogenous DNA and low cost of rich complex growth media. In this study, we describe an IMAC based method of obtaining a biologically active rBalsamin from an *E.coli* expression system. In addition, physicochemical parameters were optimised to obtain maximum expression of balsamin. Further, biophysical characterization of rBalsamin and its other functional properties were optimised.

2. Materials and methods

2.1 Enzymes, chemicals and purification kits

Seeds of *M. balsamina* were procured from National Seed Stock (India). All chemicals used in this study were either obtained from Sigma-Aldrich (St. Louis, MO, USA) or Bio-Rad Laboratories (Hercules, CA, USA) and were of analytical grade. DNA ligase, Phusion DNA polymerase and DNA markers were from New England Biolabs Inc. (Ipswich, MA, USA). Precision Plus™ dual colour protein marker was from Bio-Rad Laboratories (Hercules, CA, USA). Restriction enzymes (*BamHI* and *XhoI*) and pET- 30a(+) were procured from Novagen Inc. (Wisconsin, USA). RNAqueous® Total RNA Isolation Kit was obtained from Life Technologies Corporation (California, USA). Plasmid DNA purification kit, QIAquick PCR purification kit and QIAquick gel extraction kit were from Qiagen Pty. Ltd. (Hilden, Germany). MasterPure™ DNA Purification Kit was from Epicentre (Wisconsin, USA). Immobilized metal affinity gel (Ni^{2+} Sepharose 6 fast flow) and Hi Load™ 26/60 Superdex™ 75 preparation

grade column were procured from GE Healthcare (Little Chalfont, UK).

2.2 Bacterial strains

E.coli DH10β and BL21(DE3) strains were obtained from New England Biolabs Inc. (Ipswich, MA, USA). Bacterial strains used for antimicrobial assay were procured from American Type Culture Collection (Virginia, USA).

2.3 Molecular cloning of the gene encoding Balsamin

The genomic DNA of *M. balsamina* seeds was isolated using a MasterPure™ DNA purification kit. To amplify the balsamin (*Bal*) gene from the genomic DNA, seven different degenerate PCR primers were designed based on published data [13,14,19-23]. The list of forward and reverse primers used in the study is given in **Table 1**. The PCR reaction was performed in 50 µl volume containing; 0.5 µg of genomic DNA as template, 0.5 µM each of forward and reverse primer, 250 µM dNTP, 1.5 mM $MgCl_2$, 0.5 U Phusion DNA polymerase, and 1X Phusion HF buffer. The PCR procedure included: 98 °C for 2 min as initial denaturation, 35 cycles at 98 °C for 15 sec (denaturation), 65 °C for 30 sec (annealing), 72 °C for 60 sec (extension) and then 72 °C for 5 min as a final extension step. Finally, the PCR products were analyzed by electrophoresis on a 0.8% agarose gel.

The gene fragments obtained after PCR were purified from the gel using a QIAquick gel extraction kit. The samples (prepared by mixing 1 µg DNA template; 0.5 µM forward primer; 2.5 µl Milli Q water) were sent to Macrogen Inc., Seoul, Korea for sequencing.

2.4 Construction of recombinant vector pET-Bal

Specific sense and antisense primers were designed according to the nucleotide sequence of the balsamin: forward primer: 5'-CGGGATCCATGTTTATCAAAGC-3'; and reverse primer: 5'-CAGCTCGAGTTAGGAGCTGAAGCCT-3'. The highlighted bases in the forward and reverse primers were denoted as *BamHI* and *XhoI* restriction sites, respectively. The PCR reaction mixture was performed in 50 µl volume containing; 0.5 µg of DNA fragment (sequenced), 0.5 µM each of specific forward and reverse primer, 250 µM dNTP, 1.5 mM $MgCl_2$, 0.5 U Phusion DNA polymerase and 1X Phusion HF buffer. The PCR procedure included: 98 °C for 1 min (initial denaturation), 30 cycles at 98 °C for 10 sec, 64 °C for 20 sec, 72 °C for 45 sec and then 72 °C for 5 min as a final extension step.

The PCR amplified fragment (digested with *BamHI* and *XhoI*) was cloned into pGEM-T vector, digested with the same enzymes, and transformed into *E.coli* DH10β cells. The correct insertion of the *Bal* gene was confirmed by DNA sequencing (Macrogen, Inc., South Korea). The plasmid was double digested and subcloned between the *BamHI* and *XhoI* sites of the pET-30a(+) vector. The recombinant vector thus generated was designated pET-Bal. The correct insertion of the *Bal* gene was confirmed by colony PCR with vector-specific primers and restriction digestion with *BamHI* and *XhoI*.

2.5 Expression of rBalsamin in E.coli

For bacterial expression of the gene encoding balsamin, pET-Bal was transformed into *E.coli* BL21(DE3) competent cells. A single colony harbouring the pET-Bal recombinant plasmid was inoculated into 20 ml of LB broth containing 50 µg/ml of kanamycin and grown overnight at 37 °C and 250 rpm. The next day, the overnight culture was inoculated into 500 ml of LB broth containing 50 µg/ml of kanamycin at a ratio 1:100 and grown at 37 °C and 250 rpm until an OD_{600nm} of 0.4-0.6 was reached. Protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG). Culture parameters, such as growth temperature (20 °C, 25 °C, 30 °C and 37 °C), IPTG concentration (0.2, 0.4, 0.6, 0.8 and 1.0 mM) and culture time (4, 8, 12, 16 and 20 h) were optimized for the production of rBalsamin. After induction, the cells were harvested by centrifugation at 12,000 X g for 20 min at 4 °C. The cell pellet was dissolved in lysis buffer (50 mM Tris-HCl buffer, pH 7.5, 300 mM NaCl and 20 mM imidazole) and the cells were disrupted using an ultra-sonicator (Vibra-Cell VCX 500, Sonics, USA). Sonication was carried out for 15 min at 30% amplitude using a 3mm tapered micro tip probe. After sonication, the cells were centrifuged at 12,000 X g for 15 min at 4 °C. Samples of the soluble (supernatant) and insoluble (cell pellet) fractions were analysed by SDS-PAGE.

The insoluble fraction obtained after centrifugation was dissolved in buffer A (50 mM Tris-HCl buffer, pH 7.5, 300 mM NaCl, 20 mM imidazole and 8M urea) and stirred at room temperature for 30 min for cell disruption. The suspension was then centrifuged at 12,000 X g for 20 min at 4 °C and then passed through a 0.22 µm filter.

2.6 Purification of rBalsamin

Immobilized metal affinity chromatography (IMAC) is a common technique used to purify proteins fused to a His-tag. Since the rBalsamin included an N-terminal His-tag, therefore Ni^{2+}

Sepharose 6 fast flow was used for purification. The cell free supernatant was applied to a Ni^{2+} Sepharose 6 fast flow column (10 X 1.5 cm) pre-equilibrated with buffer A. To remove the unbound protein, the column was washed with buffer A. To refold the bound protein on the column, the column was washed with a linear gradient of urea (8-0 M) in buffer A. Finally, the bound proteins were eluted from the column using buffer B (50 mM Tris-HCl buffer, pH 7.5, 300 mM NaCl, and 250 mM imidazole). The protein content in each fraction was measured using the Bradford method and analysed on SDS-PAGE. Finally, the fractions were concentrated using an ultrafiltration membrane (Amicon Ultra-15, 10 kDa, US) and stored at -80 °C.

For further purification of rBalsamin, the concentrated fractions were applied to a Hi Load™ 26/60 Superdex™ 75 prepacked column pre-equilibrated with 50 mM Tris-HCl, pH 7.5 (degassed). The protein was eluted with 50 mM Tris-HCl, pH 7.5 (degassed) at a flow rate of 0.5 ml min^{-1}. The eluted fractions were analysed by SDS-PAGE for the presence of rBalsamin and the fractions containing the protein were concentrated by ultrafiltration, and stored in aliquots at -80 °C.

2.7 rRNA N-glycosidase activity

The assay was conducted as described by Kaur et al. [24]. Briefly, rabbit reticulocyte lysate was treated with rBalsamin for 30 min at 30 °C. After the treatment, the reaction was stopped by adding 10% SDS (w/v). Total rRNA was then extracted using RNAqueous® Total RNA Isolation Kit and aliquoted in two centrifuge tubes. One aliquot was treated with aniline acetate (pH 4.5) and the other one was left untreated. The samples were incubated at 60 °C for 3 min. The treated and untreated samples were analyzed on a 2% agarose gel and visualized using a Gel Doc™ XRS+ system (Bio-Rad, Hercules, CA, USA).

2.8 Circular dichroism (CD) spectroscopy

rBalsamin (450 μg/ml in 50 mM Tris-HCl buffer, pH 7.8) secondary structural information was derived from CD spectra obtained in the far UV range (190-260 nm) at a scanning speed of 100 nm/min using a temperature controlled Jasco J-815 CD spectrometer (Jasco Inc., Easton, MD, US).

To study the effect of temperature on the secondary structure of rBalsamin the spectra were recorded over a temperature range from 20 to 90 °C with a heating rate of 1 °C/min. In addition, to study the effect of pH, spectra of rBalsamin dissolved in buffer with pH ranging from 4-11,

were measured and analysed using Spectra manager^{TM} software.

2.9 Fluorescence spectroscopy

The fluorescence scans of rBalsamin (100 µg/ml in 50 mM Tris-HCl buffer, pH 7.8) were recorded using a spectroflurometer with parameter set as: $\lambda_{excitation}$ = 280 nm (slit width 5 nm), $\lambda_{emission}$ = 300-500 nm (slit width 5 nm), medium speed.

To study the effect of temperature on the tertiary structure of rBalsamin, the fluorescence spectrum was measured over a temperature range of 20 to 90 °C with a heating rate of 1 °C/min. Further, to study the effect of pH, the emission scans of rBalsamin dissolved in buffer with pH ranging from 4-11 were recorded.

2.10 Deoxyribonuclease (DNase)- like activity assay

DNase-like activity of rBalsamin was determined as described by Ajji et al. [10]. pUC19 plasmid (0.25 µg) was treated with various amounts of rBalsamin (0.5 µg, 1 µg, 1.5 µg or 2 µg) for 2 h at 37 °C in a final volume of 10 µl made with DNase buffer (50 mM Tris HCl, pH 7.5, 50 mM KCl, 0.1 mM $MgCl_2$). Untreated plasmid DNA and *EcoRI* treated plasmid DNA served as negative and positive controls, respectively.

2.11 Antimicrobial activity of rBalsamin

The antimicrobial activity of rBalsamin was determined by disc diffusion and broth microdilution methods, as described by Ajji et al. [10].

2.12 Antioxidant activity

2.12.1 Determination of DPPH radical scavenging activity

The activity was determined using 2, 2-Diphenyl-1-picrylhydrazyl (DPPH). For this, 40 µl of various concentrations of rBalsamin (0.1, 0.5, 1, 1.5, 2, 2.5 mg ml^{-1}) were incubated with 160 µl of 0.2 mM DPPH solution at room temperature for 30 min in the dark. After incubation, the absorbance of the samples was measured at 517 nm. The free radical scavenging activity of rBalsamin was then calculated using the formula:

% scavenging activity = Ab (control) – Ab (sample) / Ab (control) *100

2.12.2 Determination of superoxide radical scavenging activity

The activity was determined using nitroblue tetrazolium (NBT) as previously described [10].

2.12.3 Evaluation of reducing power ability

The reducing power of rBalsamin was determined according to Hong et al. [25]. Phosphate buffer and ascorbic acid were used as blank and positive controls, respectively.

3. Results and discussion

3.1 Cloning of Balsamin in E.coli

Balsamin, a type I RIP, was isolated from the seeds of *M. balsamina*. This RIP has previously shown to possess anti-HIV, anti-breast cancer, antibacterial and DNase-like activity with a potential to be used in therapeutics [9-11]. In the present study, a Balsamin encoding gene, *Bal*, was isolated from the seeds of *M. balsamina.* Since most of the RIP genes are intronless, it was feasible to obtain an open reading frame (ORF) of the *Bal* gene from genomic DNA by PCR. Therefore, genomic DNA from the seeds of *M. balsamina* was extracted (Fig. S1) and used as a template for the amplification of the gene encoding balsamin. Among the 7 degenerate primers used to amplify the *Bal* gene fragment, one primer resulted in the amplification of an ORF of the mature peptide (Fig. S2). The amplified gene (Fig. S3) was purified and sequenced. The sequencing results were confirmed by comparing the sequence with the nucleotide database using basic local alignment search tool (BLAST software, NCBI). A BLAST search of the sequenced gene showed 89% homology to α-MMC, a type-I RIP obtained from *M. charantia* (Fig. S4).

The *Bal* gene was double digested and directionally subcloned between the *BamHI* and *XhoI* sites of the pET-30a(+) vector. The recombinant plasmid thus generated was designated as pET-Bal, and transformed into *E.coli* BL 21(DE3) competent cells. The correct insertion of *Bal* gene was confirmed by colony PCR with vector specific primers (Fig. S5A) and endonucleases *BamHI* and *XhoI* digestion (Fig. S5B).

3.2 Expression and purification of rBalsamin

E.coli BL21-pET-Bal cells were cultured in LB broth containing 50 µg/ml kanamycin at 37 °C, 250 rpm until the OD_{600} reached approximately 0.6. The expression of rBalsamin was then induced by IPTG. The recombinant bacterial cells were collected by centrifugation and disrupted by sonication. The expression and molecular weight of rBalsamin was assessed in the supernatant and cell pellet by 12% SDS-PAGE. A band at the expected size (~ 29 kDa) was observed in the cell pellet of the induced cells (Fig. 1, Lane 3, arrow). No band was visible at this position in the supernatant (Fig. 1, Lane 2) indicating that rBalsamin was expressed in the insoluble fraction and possibly got accumulated in inclusion bodies rather than the soluble fraction. Also, the absence of a protein band corresponding to the rBalamin in the uninduced cells (Fig. 1, Lane 1) indicated tight repression in the absence of IPTG and fine control of rBalsamin expression in the *E.coli* BL 21(DE3) system. To obtain a maximum yield of the rBalsamin, culture conditions such as temperature (20-37 °C), induction time (4-20 h) and IPTG concentration (0.2-1.0 mM) were optimised. It was observed that the optimum expression of insoluble rBalsamin was obtained at 30 °C (Fig. 2A) after 4 h (Fig. 2B) of induction with 0.4 mM IPTG (Fig. 2C).

The His-tagged rBalsamin was purified by a two-step purification strategy. rBalsamin was expressed in the insoluble fraction, therefore, the purification of rBalsamin was carried out in a Ni^{2+} Sepaharose 6 fast flow column under denaturing conditions (buffer containing 8M urea). The rBalsamin in the cell suspension was adsorbed to the Ni^{2+} Sepharose column. After the unadsorbed proteins were washed out, refolding of the bound protein was performed by the use of a linear gradient of urea (8-0 M) in the wash buffer. Finally, the rBalsamin was eluted from the column by increasing the concentration of imidazole to 250 mM in the elution buffer (with no urea). 10 µl of sample from each of the collected fractions was loaded on a 12% SDS-PAGE gel to assess the purity of the protein (Fig. 3, Lane 5 & 6). The fractions were pooled, concentrated (Fig. 3, Lane 7) and further purified by gel filtration chromatography using a Superdex 75 gel-filtration column. The column was washed with 50 mM Tris-HCl, pH 7.5 (degassed) at a flow rate of 0.5 ml/min. Two peaks were obtained (Fig. 4) and analyzed using 12% SDS-PAGE. The purified fraction PII was subsequently found to contain most of the protein of approximately 29 kDa, corresponding to the predicted molecular mass of rBalsamin (Fig. 3, Lane 8). These fractions were pooled and concentrated. The purity of the concentrated rBalsamin was confirmed by the presence of single band on the gel (Fig. 3, Lane 9). Approximately 45 mg/L of rBalsamin was purified from culture broth by this two-step

purification procedure. A similar strategy has previously been employed for purification of other RIPs in *E.coli* strains. α-luffin, a type-I RIP, was expressed in *E. coli* BL21(DE3)pLysS strain and purified by Ni-nitrilotriacetic acid (NTA) agarose affinity chromatography. However, the final yield of soluble protein was reported to be 0.45 mg/l bacterial cell culture [20], 100 times lower compared to the protein yield in our study. α-MMC expressed in *E.coli* Rosetta (DE3)pLysS strain and purified by Ni- NTA agarose affinity chromatography produced 85 mg/l of soluble α-MMC [13], around double the yield reported in our study. This difference might be due to the fact that rBalsamin was expressed in the insoluble fraction, which required additional purification and protein-refolding steps leading to reduced protein yield.

3.3 rRNA N-glycosidase activity

RNA *N*-glycosidase activity is a unique characteristic exhibited by RIPs that causes hydrolysis of the N-glycosidic bond at A-4324 in 28S rRNA resulting in the release of a 400 bp fragment (the Endo-fragment) on aniline treatment. To confirm whether the purified rBalsamin was properly refolded during purification and is biologically active, an rRNA *N*-glycosidase assay was performed. It was observed that the 28S rRNA generated a typical Endo fragment after treatment with rBalsamin. The rRNAs seemed stable towards rBalsamin without the treatment of aniline as no fragment appeared at this stage on a 0.8% agarose gel. Only after the treatment with aniline, the rRNA was cleaved resulting in generation of the Endo-fragment (Fig. 5). Native balsamin (nBalsamin) isolated from *M. balsamina* served as a positive control. These results were in accordance with RNA *N*-glycosidase activity of other RIPs reported from different plant species [26,27].

3.4 Biophysical characterization of rBalsamin

The secondary structure of rBalsamin expressed in the *E.coli* expression system was determined using CD spectroscopy. The CD spectrum of the rBalsamin in the far UV region indicated the presence of predominantly α-helices and random coils. rBalsamin exhibited 34.8% α-helix, 19.3% β-sheet, 12.5% turn and 32.5% random coils at 25 °C, which were comparable to the CD profile reported for nBalsamin purified from *M. balsamina* [24]. Some similar structures have been reported with other RIPs. Trichosanthin (TCS), a type-I RIP purified from *Trichosanthes kirilowii* maxim, comprises of α-helices, β-sheets, random coils and turns where the percentage of secondary structure confirmation varied with change in pH [28].

The secondary structure of rBalsamin was found to be stable in the temperature range of 20-60 °C as little or no change in the CD spectrum was observed within this temperature range. However, above 60 °C, a slight distortion was observed in the secondary structure indicating that rBalsamin is unstable at high temperatures (Fig. 6A). Also, the effect of pH on the secondary structure of rBalsamin was studied to determine the stability of the protein. rBalsamin was stable as no spectral variations were noted between pH 6-11. However, below pH 6, a slight disruption in the CD spectra was observed indicating that rBalsamin is unstable under acidic conditions (Fig. 6B).

Temperature and pH-dependence effects on the fluorescence emission spectra of rBalsamin were studied using fluorescence spectroscopy. rBalsamin was found to be stable in the temperature range of 20-60 °C as no shift in the emission peak was observed within this temperature range. However, above 60 °C, a decrease in the fluorescence intensity with a shift in emission peak was observed, indicating that rBalsamin is unstable at higher temperatures (Fig. 7A). The fluorescence scans in the pH range of 4 to 11 demonstrated that rBalsamin is stable in a pH range of 6 to 9 as no shift in the emission peak was observed within this range. However, an increase in the fluorescence intensity below pH 6 and decrease in the fluorescence intensity above pH 9 with a shift in the emission peak towards higher wavelength suggested that rBalsamin is unstable at pH below 6 and above 9 (Fig. 7B).

3.5 DNase-like activity of rBalsamin

To optimize rBalsamin concentration for DNase-like activity, pUC19 plasmid was used as a substrate. Treatment of supercoiled pUC19 with various concentrations of rBalsamin (0.5-2 μg) resulted in conversion of supercoiled plasmid into the open circular and linear forms (Fig. 8). 0.5 and 1 μg of rBalsamin treatment partially converted the supercoiled form into the open circular form. However, when the concentration of rBalsamin was increased to 1.5 μg, almost complete disappearance of supercoiled plasmid was observed with the appearance of the linear form of the DNA. At a concentration of 2 μg, rBalsamin completely converted supercoiled and open circular forms of DNA to the linear form, suggesting that 2 μg of rBalsamin is the optimum concentration for DNase-like activity (Fig. 8). These results were in accordance with DNase-like activity of other RIPs reported from different plant species [29] and nBalsamin from *M. balsamina* [10].

3.6 Antimicrobial activity of rBalsamin

RIPs have been classified as defense proteins, which could function as antimicrobial agents [30]. ME1 and ME2, two type I RIPs, from *Mirabilis expansa* roots exhibited strong inhibitory activity against an array of fungal pathogens including *Fusarium* and *Trichoderma* species [31]. Similarly, α-MMC from *M. charantia* demonstrated growth inhibitory effect against various bacterial and fungal pathogens such as *Fusarium* species, *E. coli, P. aeruginosa, S. aureus and Bacillus subtilis* [13]. In this study, disc diffusion method was performed to determine the zone of inhibition of different dilutions of rBalsamin on various pathogenic microorganisms. The assay demonstrated that rBalsamin inhibited the growth of pathogens in a dose dependent manner with maximum inhibitory effect observed at the highest dose. rBalsamin was found to be highly effective against *S. epidermidis*, *S. aureus*, *S. enterica* and *E. coli* and comparatively less effective against *P. aeruginosa* and *E. faecalis* (Fig. S6). The zone of inhibition caused by different dilutions of rBalsamin on the various pathogens ranged between 2.2-9.0 mm (Table 2).

Further, the minimum inhibitory concentrations (MIC's) of rBalsamin were determined by the broth microdilution method. The MIC's of rBalsamin were found to be in the following order: *S. epidermidis* < *S. aureus* < *S. enterica* = *E. coli* < *P. aeruginosa* = *E. faecalis* (Fig. S7, Table 3) suggesting that rBalsamin exhibits growth inhibitory activity towards various pathogens that could prove beneficial in plant protection.

3.7 Antioxidant activity of rBalsamin

DPPH (2,2-diphenyl-1-picrylhydrazyl) is a free radical that is widely used to monitor chemical reactions involving free radicals [32-34]. DPPH has a violet colour in the solution with a characteristic absorption at 517 nm. In presence of an antioxidant molecule, it gets reduced and becomes colourless or pale yellow. However, in our study, rBalsamin concentration did not affect free radical scavenging of DPPH as no DPPH reduction was observed (Fig. S8A).

The superoxide radical scavenging assay is based on the capacity of the protein to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) [35]. However, in our study, it was observed that rBalsamin did not exhibit this activity as no NBT reduction was observed with different concentrations of rBalsamin (Fig. S8B).

Reducing power measures the total antioxidant ability of a compound and the efficiency of an antioxidant to donate electrons. The reducing ability of the compound ferricyanide complex (Fe^{3+}) to ferrous serves as an important indicator of antioxidant activity [36]. The

assay showed that the reducing power ability tended to increase as the concentration of rBalsamin increased. However, none of these results were statistically significant demonstrating that the protein does not possess reducing ability (Fig. S8C).

4. Conclusion

In this study, we discussed the development of a two-step purification procedure for the production of functionally bioactive balsamin from *M. balsamina*. Optimization of expression parameters such as induction time, IPTG concentration and temperature led to maximum protein yield after 4 h of 0.4 mM IPTG induction at 30 °C in the *E.coli* BL21(DE3) expression system. The incorporation of a His-tag on the N-terminus of the protein allowed purification of rBalsamin by Ni^{2+} Sepharose affinity chromatography. Functional analysis indicated that rBalsamin exhibited RNA *N*-glycosidase, DNase-like and antimicrobial activity in a concentration-dependent manner. The structure of rBalsamin was found to be sensitive to higher temperatures, and a highly acidic or basic environment. Heterologous expression of rBalsamin using *E.coli* system not only provides an alternative approach for large-scale production and purification of rBalsamin, which would enable further structural and functional studies and detailed investigation on the mechanism of DNase-like and antimicrobial activities of balsamin, but also a versatile approach for genetic manipulation in the rational development of this potential therapeutic agent.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Legend to Figures:

Fig. 1 SDS-PAGE analysis of expression of rBalsamin in *E.coli*.

Fig. 2 Optimization of expression of rBalsamin in *E.coli*.

Fig. 3 SDS-PAGE analysis of rBalsamin expressed in *E.coli*.

Tables:

Table 1 Degenerate primers used for balsamin gene amplification.

Gene	Primer sequence
MAP	**Forward primer: 5'**-CATGCGGGATCCATGGTGAAATGCTTAC-**3'** **Reverse primer: 5'**-CGAGCCTCGAGTCAATTCACAACAG-**3'**
Gel	**Forward primer: 5'**-CATGCGGGATCCACYTGRTCNACNGCNGTNACRTARTAYTTYTT-**3'** **Reverse primer: 5'**-CGAGCCTCGAGTTYAARGAYGCNCCNGAYGCNGCNTAYGARGG-**3'**
Sap	**Forward primer: 5'**-CATGCGGGATCCCTGCAGAATTCGCATGGATCCTGCAAT-**3'** **Reverse primer: 5'**-CGAGCCTCGAGCTGCAGAATTCGCCTCGTTTGACTACTTTG-**3'**
Luf	**Forward primer: 5'**-CATGCGGGATCCCAGATGTGAGGTTCAGTTTGTCAGGT-**3'** **Reverse primer: 5'**-CGAGCCTCGAGCGCAACATTTTGTTTGTA-**3'**
PAP	**Forward primer: 5'**-CATGCGTCCCTTCAGCTGATAAATACGATCACCTTTGATC-**3'** **Reverse primer: 5'**-CGAGCCTCGAGCGGGATCCGAATCCTTCAAATAGATCAC-**3'**
Mom	**Forward primer: 5'**-CATGCGGGATCCGATGTTAGCTTTCGTTTG-**3'** **Reverse primer: 5'**-CGAGCCTCGAGTTAGGGCATTCTAGTAGCTC-**3'**
PAPII	**Forward primer: 5'**-CATGCGGGATCCATRTAYTTRAATCTIGCIGCYT-**3'** **Reverse primer: 5'**-CGAGCCTCGAGTGAAYATHGTITTYGAYGTIGARAA-**3'**

***MAP*: *Momordica* antiviral protein; *gel*: gelonin; *sap*: saporin; *Luf*: α-luffin; *PAP*: pokeweed antiviral protein; *Mom*: α –momorcharin and *PAPII*: pokeweed antiviral protein II.**

Table 2 Antibacterial activity of rBalsamin towards different pathogenic microorganisms.

Microorganisms	Zone of inhibition in mm ± SD		
	rBalsamin concentration (µg/ml)		
	50	100	200
Staphylococcus aureus	3.1 ± 0.2	6.2 ± 0.5	8.0 ± 0.4
Pseudomonas aeruginosa	ND	2.2 ± 0.3	5.1 ± 0.7
Escherichia coli	2.2 ± 0.1	4.4 ± 0.3	6.3 ± 0.5
Staphylococcus epidermidis	4.1 ± 0.3	6.2 ± 0.4	9.0 ± 0.7
Salmonella enterica	2.7 ± 0.2	5.5 ± 0.6	7.1 ± 0.5
Enterococcus faecalis	ND	ND	3.3 ± 0.2

Values are mean ± SD based on three sets of experiments; ND denotes not detected.

Table 3 Minimum inhibitory concentrations (MICs) of rBalsamin against different pathogenic microorganisms.

Microorganisms	MICs of rBalsamin (μg/ml)
Staphylococcus aureus	6.25
Pseudomonas aeruginosa	25.00
Escherichia coli	12.50
Staphylococcus epidermidis	1.56
Salmonella enterica	12.50
Enterococcus faecalis	25.00

Highlights:

- Balsamin was cloned and expressed in *E.coli* BL21 (DE3) strain, and purified by two-step purification
- Recombinant Balsamin exhibited RNA *N*-glycosidase and DNase-like activity.
- rBalsamin consisted of α-helical and random coiled with minimal turns and β-sheets.
- Structure of rBalsamin was found to be sensitive to higher temperatures, acidic/ basic environment.
- Antimicrobial assay revealed that rBalsamin inhibited the growth of various bacterial pathogens in a dose dependent manner

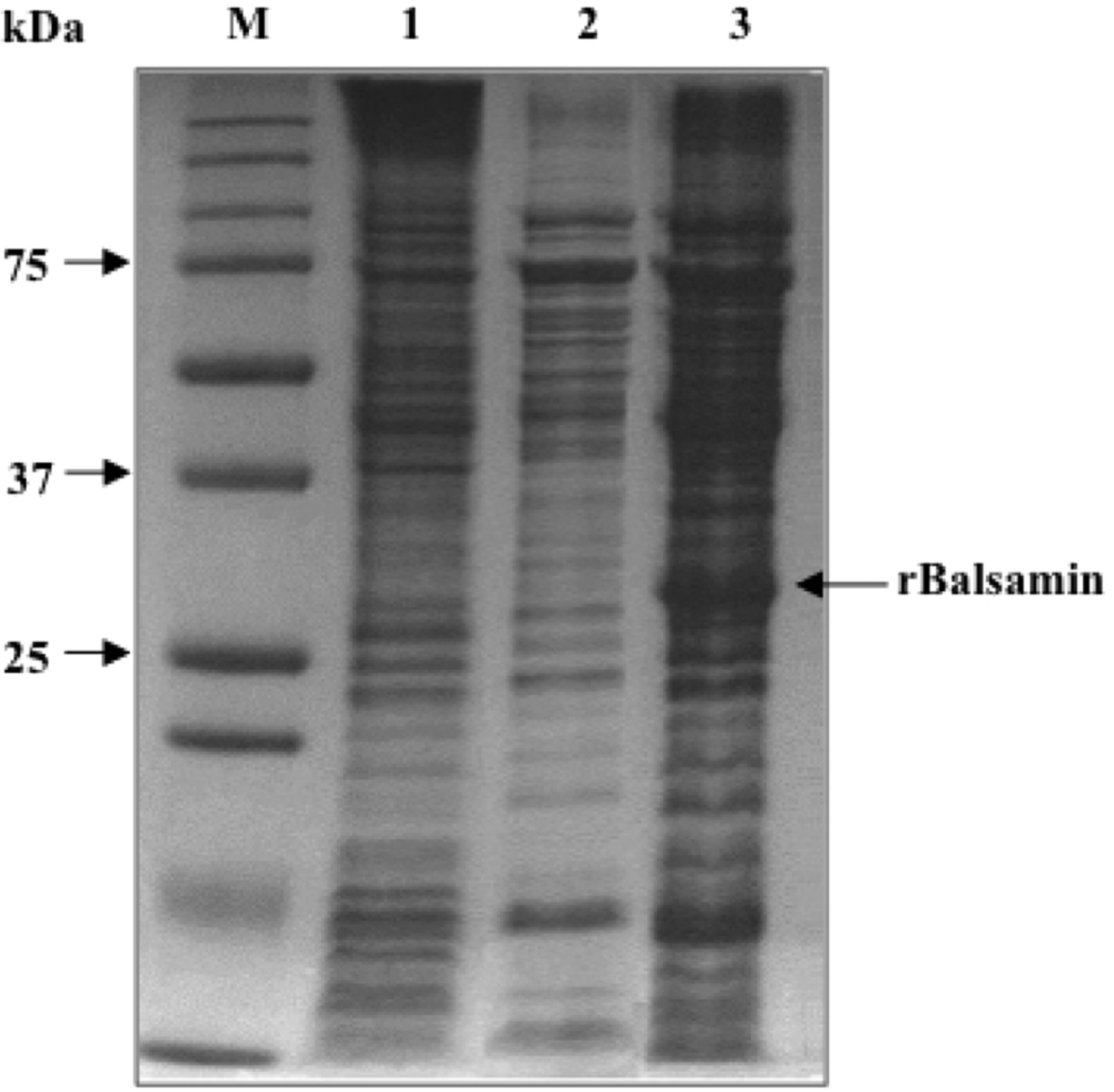

Figure 1

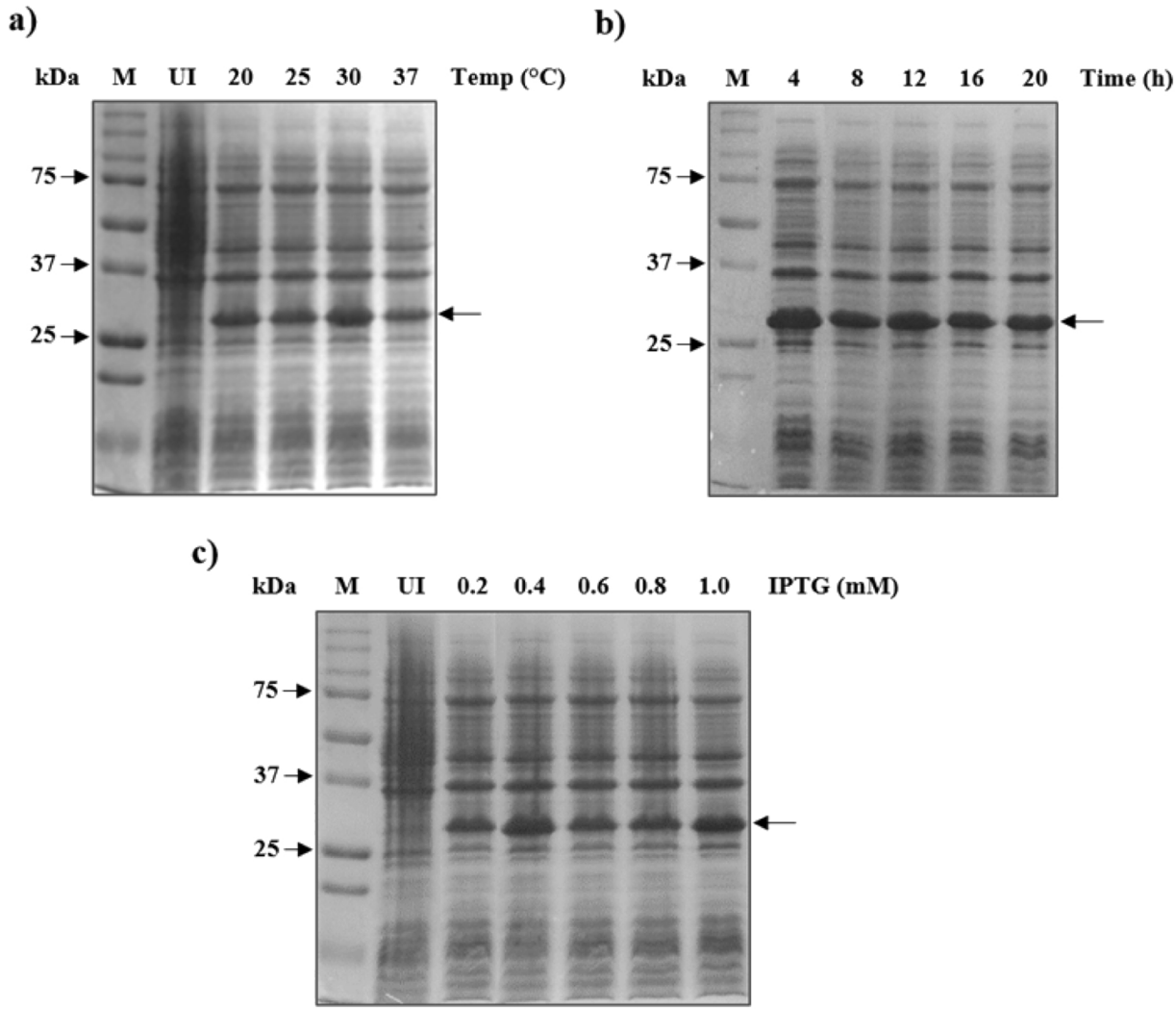
a)
kDa
M
UI
20
25
30
37
Temp (°C)
75
37
25
b)
kDa
M
4
8
12
16
20
Time (h)
75
37
25
c)
kDa
M
UI
0.2
0.4
0.6
0.8
1.0
IPTG (mM)
75
37
25

Figure 2

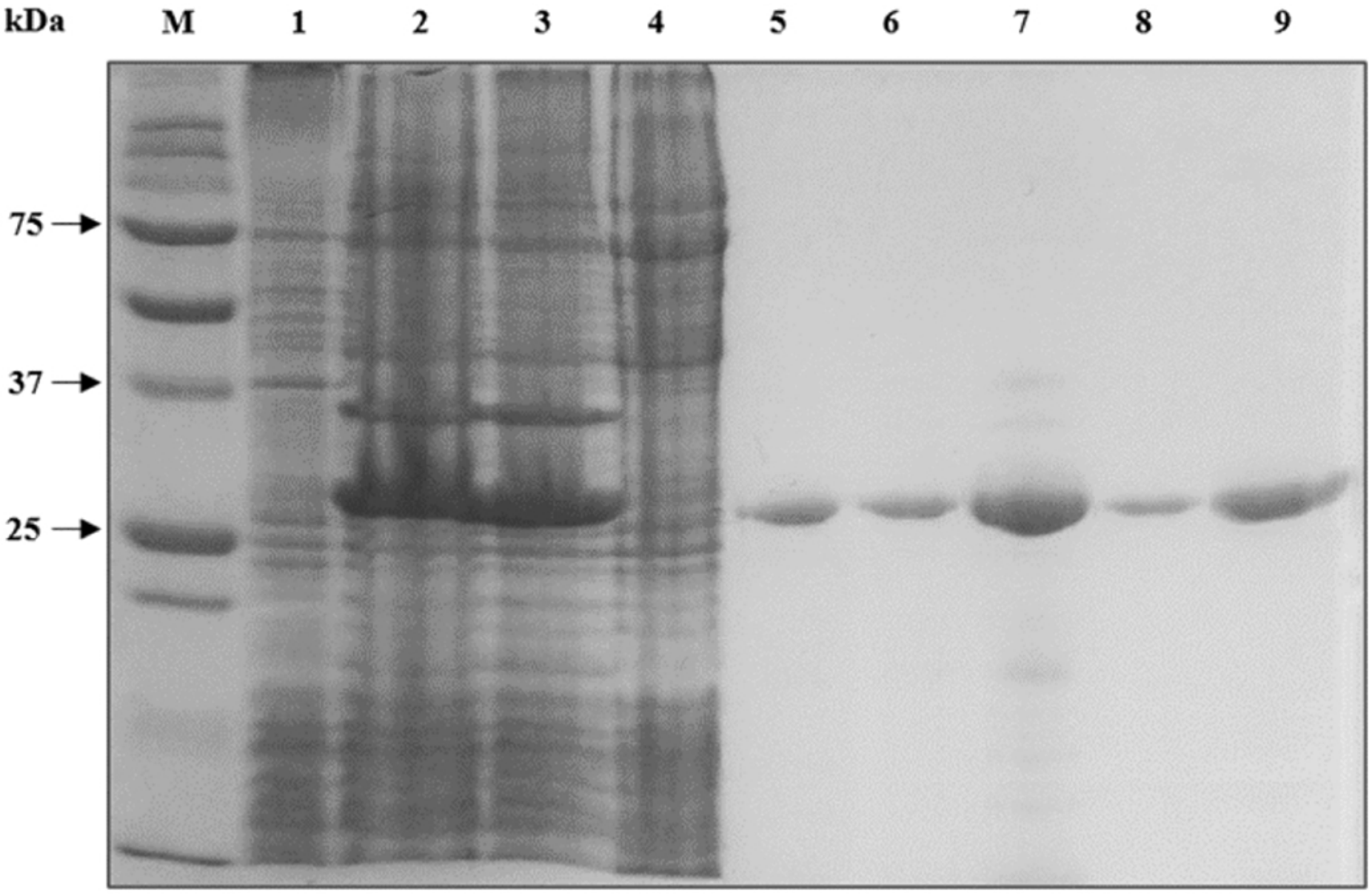

Figure 3

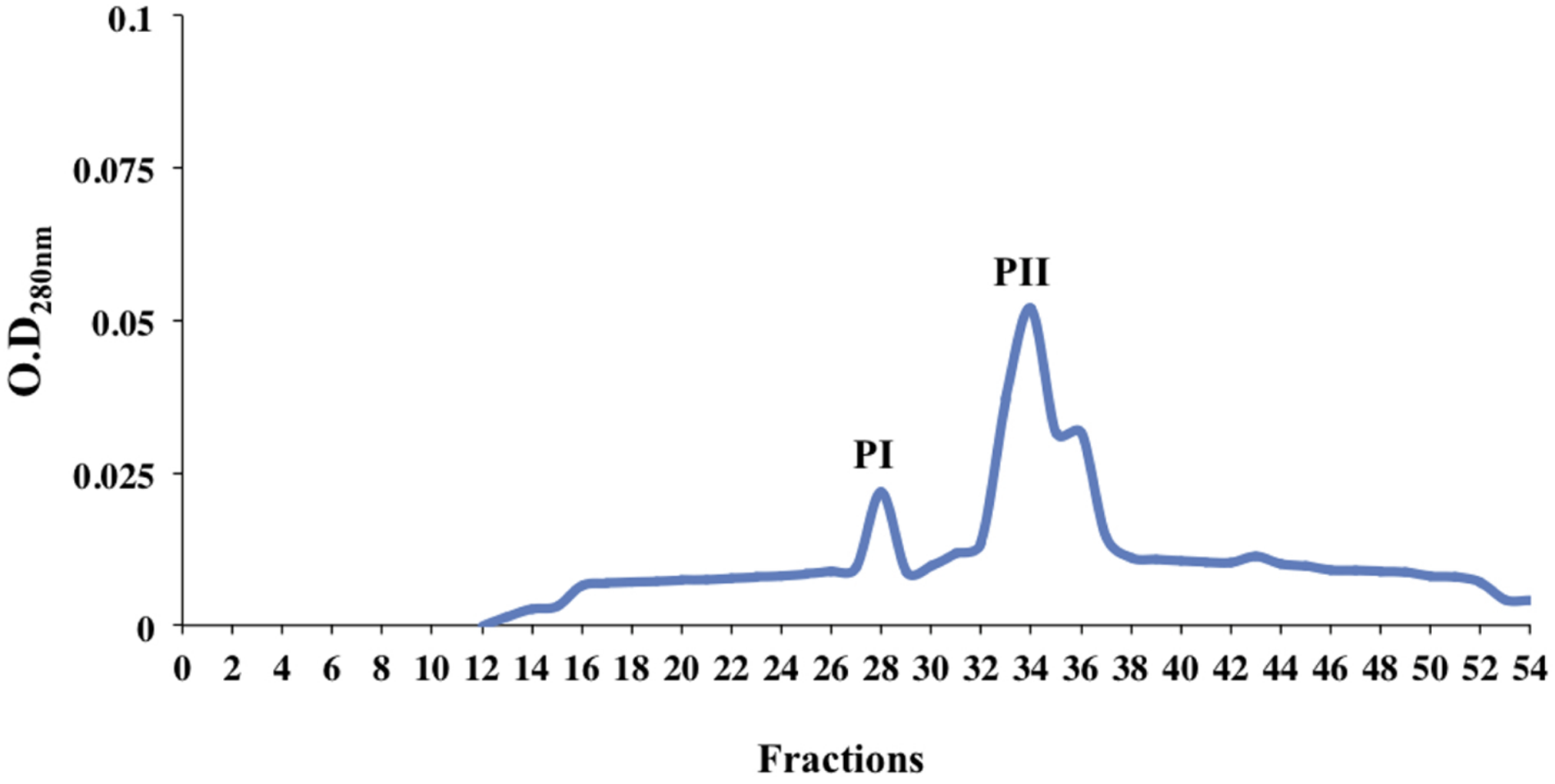
0.1
0.075
0.05
0.025
0
O.D280nm
PI
PII
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54
Fractions

Figure 4

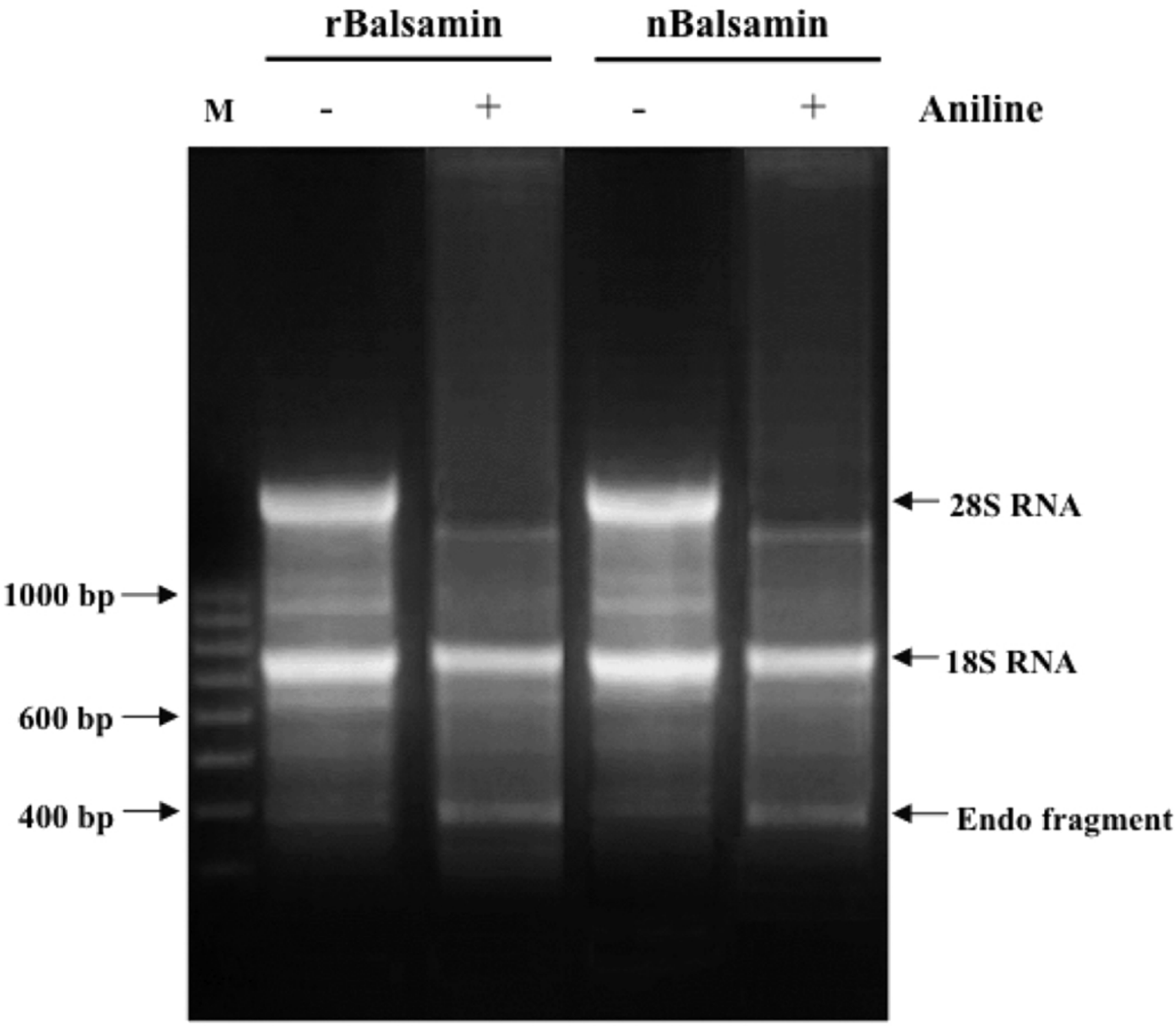
rBalsamin
nBalsamin
M
-
+
-
+
Aniline
28S RNA
18S RNA
Endo fragment
1000 bp
600 bp
400 bp

Figure 5

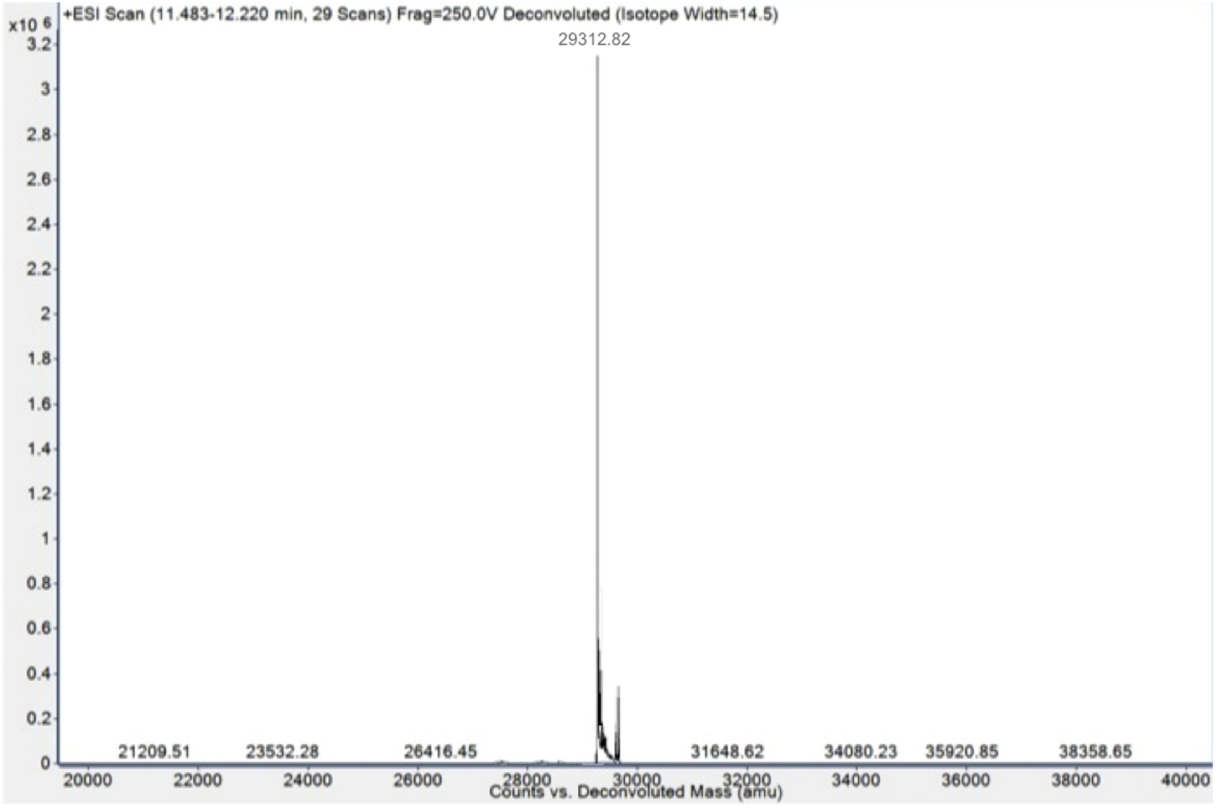
+ESI Scan (11.483-12.220 min, 29 Scans) Frag=250.0V Deconvoluted (Isotope Width=14.5)
x10 6
29312.82
21209.51
23532.28
26416.45
31648.62
34080.23
35920.85
38358.65
Counts vs. Deconvoluted Mass (amu)

Figure 6

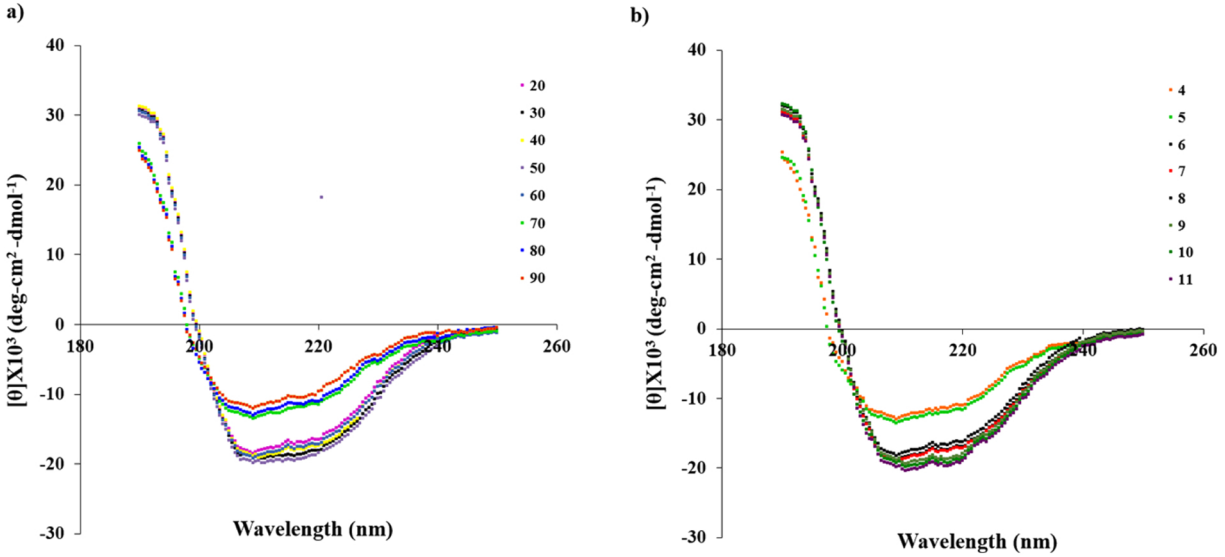
a)
40
30
20
10
0
-10
-20
-30
180
200
220
240
260
[θ]X10³ (deg-cm² -dmol⁻¹)
Wavelength (nm)
20
30
40
50
60
70
80
90
b)
40
30
20
10
0
-10
-20
-30
180
200
220
240
260
[θ]X10³ (deg-cm² -dmol⁻¹)
Wavelength (nm)
4
5
6
7
8
9
10
11

Figure 7

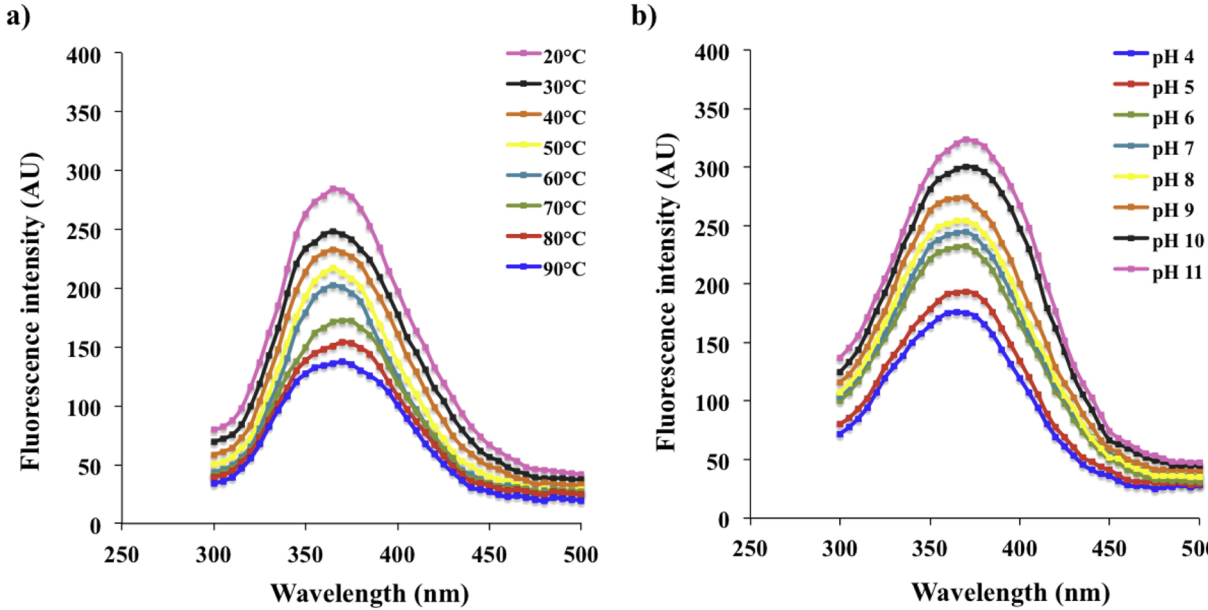
a)
Fluorescence intensity (AU)
Wavelength (nm)
20°C
30°C
40°C
50°C
60°C
70°C
80°C
90°C
b)
Fluorescence intensity (AU)
Wavelength (nm)
pH 4
pH 5
pH 6
pH 7
pH 8
pH 9
pH 10
pH 11

Figure 8

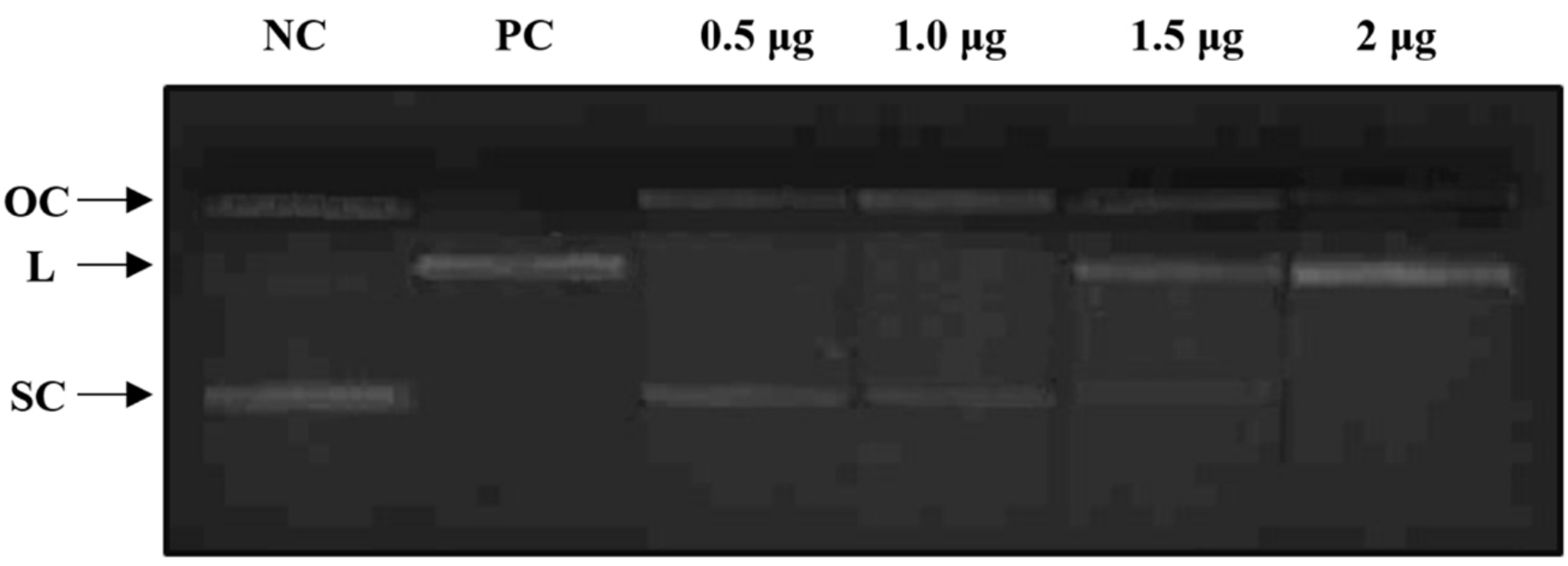

Figure 9